The role of UBL domains in ubiquitin-specific proteases

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Abstract

Ubiquitin conjugation and deconjugation provides a powerful signalling system to change the fate of its target enzymes. Ubiquitination levels are organized through a balance between ubiquitinating E1, E2 and E3 enzymes and deubiquitination by DUBs (deubiquitinating enzymes). These enzymes are tightly regulated to control their activity. In the present article, we discuss the different ways in which DUBs of the USP (ubiquitin-specific protease) family are regulated by internal domains with a UBL (ubiquitin-like) fold. The UBL domain in USP14 is important for its localization at the proteasome, which enhances catalysis. In contrast, a UBL domain in USP4 binds to the catalytic domain and competes with ubiquitin binding. In this process, the UBL domain mimics ubiquitin and partially inhibits catalysis. In USP7, there are five consecutive UBL domains, of which the last two affect catalytic activity. Surprisingly, they do not act like ubiquitin and activate catalysis rather than inhibiting it. These C-terminal UBL domains promote a conformational change that allows ubiquitin binding and organizes the catalytic centre. Thus it seems that UBL domains have different functions in different USPs. Other proteins can modulate the roles of UBL domains in USP4 and USP7. On one hand, the inhibition of USP4 can be relieved when the UBL is sequestered by another USP. On the other, the activation of USP7 is increased, when the UBL-activated state is stabilized by allosteric binding of GMP synthetase. Altogether, UBL domains appear to be able to regulate catalytic activity in USPs, but they can use widely different mechanisms of action, in which they may, as in USP4, or may not, as in USP7, use the direct resemblance to ubiquitin.

Introduction

Ubiquitin conjugation is a post-translational modification where a 76-amino-acid ubiquitin protein is conjugated to a lysine residue on a target protein. Since ubiquitin itself has seven lysine residues, this results in a plethora of signals with roles in different cellular processes [1]. In practice, ubiquitination is important in just about any cellular event, from endocytosis to transcription, and from DNA repair to the cell cycle.

Because of these critical cellular roles, the ubiquitination event is balanced by deubiquitination. There are five classes of DUBs (deubiquitinating enzymes) that hydrolyse the isopeptide bond between ubiquitin and its target [2,3]. The USP (ubiquitin-specific protease) family is the largest family of DUBs, with approximately 85 members [2].

To control the necessary levels of ubiquitination, DUBs need to be tightly regulated, and this is organized in a variety of ways. Such regulation can affect the recruitment to the target, the specificity to different ubiquitin chain types or the intrinsic catalysis. It can be organized through post-translational modifications, through external modulating proteins, through the substrate and through internal domains within the DUB protein [3–6].

Interestingly, several members of the USP family of DUBs contain UBL (ubiquitin-like) domains. These protein domains are often found in multidomain proteins. They share the β-grasp fold with ubiquitin, although they lack the terminal glycine residues that are required for conjugation to a target lysine residue. Some UBLs have clear sequence similarity to ubiquitin and others do not. The latter are therefore sometimes referred to as UFDs (ubiquitin-fold domains) (K. Hofmann, personal communication).

So far, several different families of UBL domains have been described [7]. They are present in proteasomal shuttle factors such as Rad23 and Dsk2, where they are believed to play a role in recruitment of ubiquitinated proteins to the proteasome [8,9]. Integrated UBL domains in other proteins such as parkin can also be recruited to the proteasome [10]. Recruitment, in this case to DUB targets, is also seen as the function of the UBL domain in the USP1 activator UAF1 (USP1-associated factor 1) [11]. However, UBL domains also play roles in the enzymatic activity of certain immune-response-inducible kinases, such as IKKβ (inhibitor of nuclear factor κB kinase β) [12].

The first UBL domain in the USP family was identified in USP14 by Shi and co-workers [13]. Subsequently, a bioinformatics analysis revealed that related β-grasp fold domains exist in at least 16 USPs [14] (Figure 1A). Given their

Key words: deubiquitinating enzyme (DUB), GMP synthetase (GMPS), proteasome, ubiquitin-like domain (UBL domain), ubiquitin-specific protease.

Abbreviations used: AMC, 7-amino-4-methylcoumarin; DUB, deubiquitinating enzyme; DUSP, dual-specificity phosphatase; GMPS, GMP synthetase; HAUSP, herpesvirus-associated ubiquitin-specific peptidase; HUBL, HAUSP UBL; MDM2, murine double minute 2; SAXS, small-angle X-ray scattering; TNFα, tumour necrosis factor α; UBL, ubiquitin-like; UFD, ubiquitin-fold domain; USP, ubiquitin-specific protease; UAF1, USP1-associated factor 1.

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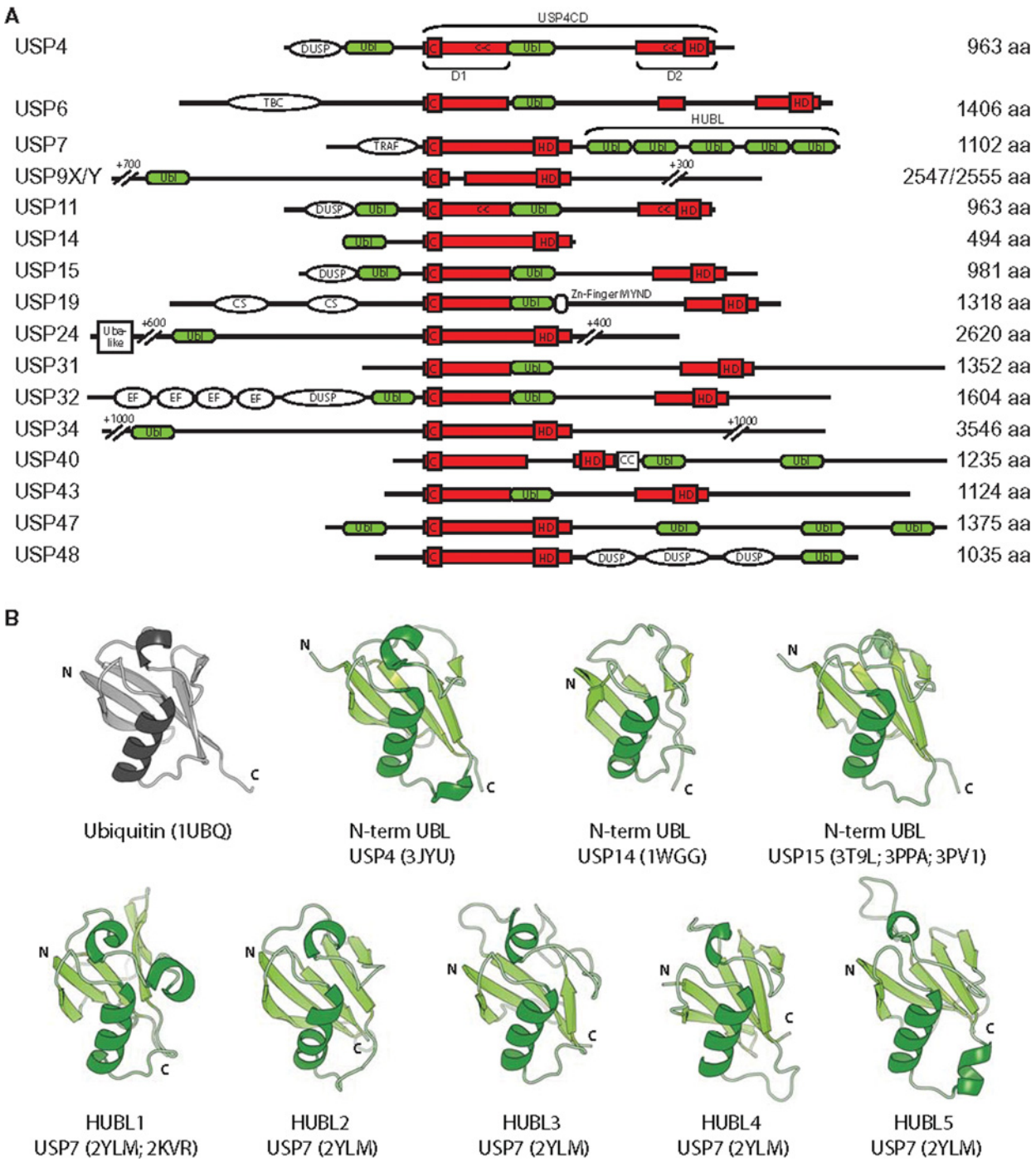
Figure 1 | **USPs contain UBL domains**

(**A**) Several USPs contain one or more UFDs embedded in the sequence at different locations with respect to the catalytic domain [14]. (**B**) Three-dimensional structures have been resolved for several UBL domains in USPs, revealing that they indeed fold like ubiquitin. If more than one structure is known, all PDB identifiers are given, with the first structure shown. aa, amino acids; TRAF, tumour-necrosis-factor-receptor-associated factor.

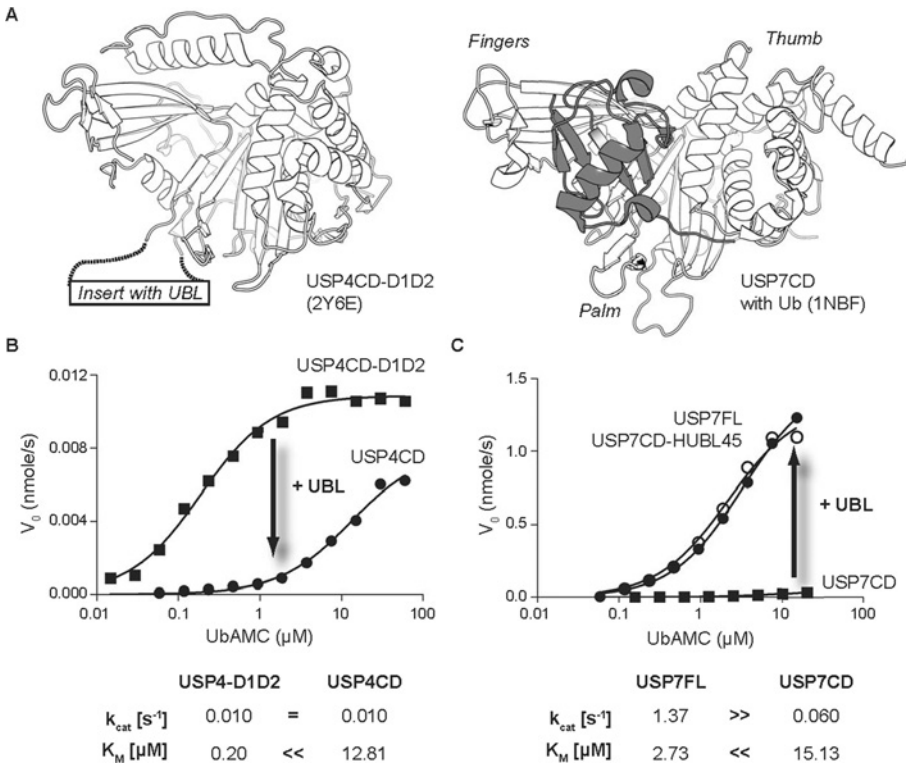


low sequence homology with ubiquitin, they really should be called UFDs. In practice, however, they have been named UBL domains and we will maintain this nomenclature in the present article.

Despite the lack of sequence similarity among each other, they share common features, since, e.g., a PSI-BLAST analysis of any member of this subfamily will find the other UBL domains in USPs efficiently, but not the UBL

Figure 2 | UBL domains inhibit USP4, but activate USP7 catalysis

(**A**) USPs have a common catalytic domain, here shown in cartoon representation for the D1D2 fragment of USP4 (see Figure 1) [29] (left) and for USP7 in complex with ubiquitin [44] (right). The insert containing the UBL domain of USP4 is indicated. (**B**) The UBL domain in USP4 inhibits catalytic activity in ubiquitin–AMC assays, whereas (**C**) the UBL domains in USP7 strongly activate catalysis.



	USP4-D1D2		USP4CD
k_{cat} [s^{-1}]	0.010	=	0.010
K_M [μM]	0.20	<<	12.81

	USP7FL		USP7CD
k_{cat} [s^{-1}]	1.37	>>	0.060
K_M [μM]	2.73	<<	15.13

domains in unrelated proteins. Several structures of these UBL domains have been resolved (Figure 1B), and a structural alignment shows 1–3 Å (1 Å = 0.1 nm) root mean square deviations.

The USP family of DUBs has a common catalytic domain (Figure 2A) in which a hand-like domain holds on to ubiquitin, guiding the ubiquitin C-terminus to the catalytic triad of the papain-like cysteine protease family. The overall length of the catalytic domain of the USP family members varies widely, owing to insertions at five different sites [5]. The UBL domains are inserted at different sites relative to the catalytic domain (Figure 1A), N-terminally to, inserted into or C-terminally to the catalytic domain

It was found that these UBL domains can play widely different roles in the regulation of their DUB activity. In the present article, we compare the function of the three UBL domains that have been described, and their widely different activities in recruitment, inhibition or activation.

USP14: recruitment to the proteasome

USP14 is localized at the proteasome [15], where it plays a special role in rescuing proteins from degradation by removing ubiquitin [13,16]. This release of ubiquitin spares it from degradation, minimizing fluctuations in the free ubiquitin pool. Loss of USP14 leads to ataxia [17] and reduced levels of ubiquitin [18].

USP14 has an N-terminal UBL domain (Figure 1B). The crystal structure of the catalytic domain shows a catalytic triad correctly folded for catalysis, but a number of blocking loops that prevent access to the ubiquitin-binding site [13]. Biochemical analysis showed that the UBL domain in USP14 is important for its recruitment to the proteasome [13]. The proteasome interaction is critical for efficient catalysis by USP14, presumably by rearranging the blocking loops [13]. The primary role of the UBL domain appears to be in recruitment to the proteasome, similar to roles described for UBL domains in Rad23 and parkin [9,10].

USP4: inhibiting catalysis

USP4 was known previously as UNP (ubiquitous nuclear protein) [19]. Identified as a proto-oncogene related to Tre2/Tre17 (USP6), USP4 shows a consistently elevated gene expression level in small cell tumours and lung adenocarcinomas, suggesting that it may have a causative role in neoplasia [20]. Besides possible roles in TNFα (tumour necrosis factor α) [21] and Wnt signalling [22], as well as

recruitment to the adenosine A_{2A} receptor [23], USP4 is recruited to the spliceosome by complex formation with Sart3 [24]. Here, it preferentially deubiquitinates Lys^{63}-linked chains on the U4 component Prp3. Another component of the spliceosome complex is the catalytically inactive USP39 [24], which controls the mRNA levels of Aurora B [25]. More recently, Zhang et al. [26] have shown how USP4 stabilizes ARF-BP1 (alternative reading frame-binding protein 1) and the subsequent reduction in p53 levels [26].

There are two homologues of USP4, namely USP11 and USP15. These USPs have a common domain structure and high sequence similarity (47–57 % identity), but very different biological functions. Each of them has an N-terminal DUSP (domain present in ubiquitin-specific protease)-UBL domain combination, and several crystal structures of these have been solved showing differences mainly in the relative orientation of the DUSP and UBL domains and in the way the finger loop connecting the two domains packs against the UBL domain [27,28]. The function of these DUSP-UBL domains in the full-length protein is not yet understood but given the significant differences in surface characteristics between ubiquitin and the UBL domain, it is unlikely that the USP15 N-terminal UBL domain may act as ubiquitin. To date, the only functional role of the DUSP-UBL domain is to mediate the interaction between USP4 and Sart3 [24].

The catalytic domain of USP4 contains a large insert into which a UBL domain is embedded in a larger unstructured region. Removal of this insert by proteolysis or by mutagenesis results in the canonical USP catalytic domain (Figure 2A) split into two domains named D1 and D2 [29] (Figure 1A). Its crystal structure is highly similar to most other USP catalytic domain structures with a correctly aligned catalytic centre [29]. As in USP14, rearrangement of a number of blocking loops is required to allow access of ubiquitin to the binding site in this catalytic domain.

The role of the UBL domain inserted in the catalytic domain became clear upon comparison of the catalytic activity of the full catalytic domain with that of the D1D2 fragment, lacking the UBL insert. In these assays, the D1D2 fragment was much more efficient against a minimal fluorigenic ubiquitin substrate (Figure 2B) or against di-ubiquitin [29], indicating that the UBL insert inhibits activity.

Kinetic analysis of the enzymatic activity revealed differences, primarily at the K_m level, suggesting that the insert acts as a competitive inhibitor. Indeed, binding studies revealed an affinity of $\sim$1 μM between the UBL domain and the D1D2 catalytic domain.

Since the UBL domain binds so well, a strong inhibition of ubiquitin binding is expected when the insert is present in the catalytic domain. In practice, however, the difference is not that extreme. Also the K_i for the UBL domain against D1D2, as determined from a Dixon-type analysis where kinetic data are measured at different inhibitor concentrations, was found to be $\sim$45 μM, substantially higher than the binding constant. Hence further regulation of inhibition must take place, possibly through conformational changes.

In USP4, the UBL domain competes with ubiquitin for its binding site in the USP hand and this inhibits the catalytic activity. Importantly, the current data do not answer the question of whether the UBL domain adopts the same conformation in the USP4-binding site. Since there is no sequence similarity to ubiquitin, it could bind in any orientation in the USP4 catalytic site, but in any case it does compete directly with ubiquitin for this binding site.

USP7: activating catalysis

USP7/HAUSP (herpesvirus-associated ubiquitin-specific peptidase) has many roles in controlling critical proteins during the stress response [30]. It was first identified as a DUB for p53, but later found to also control auto-ubiquitination, and thus stability, of the E3 ligase of p53, MDM2 (murine double minute 2). Dependent on external conditions and regulators such as TSPYL5 (testis-specific Y-encoded-like protein 5) [31], it can thus either activate or inactivate p53 function. A number of other important targets have been resolved, including PTEN (phosphatase and tensin homologue deleted on chromosome 10) [32] and FOXO4 (forkhead box O4) [33]. In other reports, USP7 was shown to regulate claspin levels, affecting the DNA-damage checkpoint [34] and to alter epigenetic responses through regulation of DNMT1 [DNA (cytosine-5-)-methyltransferase 1] and PRC1 (Polycomb-repressive complex 1) [35,36]

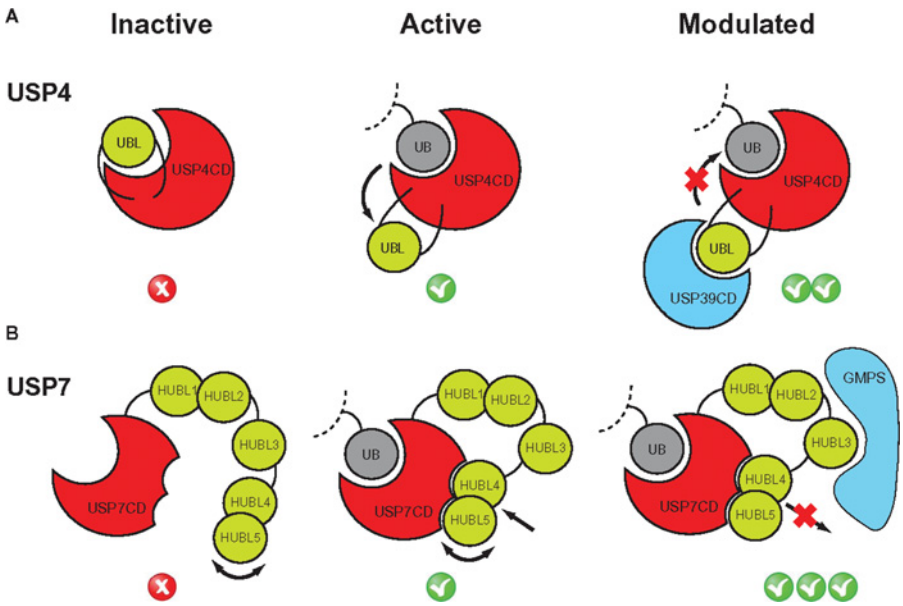
USP7 is an essential gene [37] and even specific knockout in brain cells leads to neonatal lethality [38]. USP7 is up-regulated in prostate cancer [32] and relates directly to tumour aggressiveness, linking it to an important role in non-small-cell lung carcinogenesis [39]. In a colon cancer xenograft model, both up- and down-regulation of USP7/HAUSP inhibit tumour growth in the absence of stress [40]. Altogether, USP7 is thought to be an attractive target for cancer therapy [30,41,42].

USP7 consists of an N-terminal TRAF (tumour-necrosis-factor-receptor-associated factor) domain that is important for recruitment to the target proteins [13,43]. It also has a minimal catalytic domain, that was shown to adopt an inactive state, where the catalytic triad is misaligned [44]. However, in the presence of covalently bound ubiquitin, this site is reorganized to the active triad as found in other USPs, such as USP14 and USP4 [44]. Interestingly, USP7 has a 64 kDa C-terminal domain that contains five UBL domains. This domain has been named the HUBL domain (for HAUSP UBL domain) [45].

Analysis of the USP7 function showed that the C-terminal HUBL domain is required for full activity of the enzyme [45–47]. The crystal structure of this domain revealed that the five UBL domains are arranged in an extended $2+1+2$ fold, where the dimers share a distinct hydrophobic core [45]. SAXS (small-angle X-ray scattering) analysis showed that despite some flexibility, the extended state occurs in solution, but, in the full-length protein, the HUBL domain folds back on to the catalytic domain.

Figure 3 | Model for UBL domain function in USP catalysis and modulation by external regulators

(**A**) USP4 is inhibited by its UBL domain: it competes with ubiquitin for binding to the active site. This can be relieved by other USPs binding to the inhibitory UBL domain [29]. (**B**) USP7 is activated by its UBL domains. Binding of the C-terminal peptide and last two UBL domains, HUBL45, promotes ubiquitin binding and rearrangement of the catalytic triad into its active state. This active state, with HUBL45 bound, is allosterically promoted by GMPS binding to the first three UBL domains, HUBL123 [45].



How the HUBL domain activates USP7 was studied in detail [45]. Mapping studies showed that the two C-terminal UBL domains are sufficient for full activation of the catalytic domain. The very C-terminal peptide after the last UBL is necessary, but not sufficient, for this activation, and it requires the HUBL-45 di-UBL unit for binding to the catalytic domain. This binding promotes ubiquitin binding, improving it from virtually undetectable to 1 μM affinity. It also improves catalysis, presumably by helping to rearrange the catalytic triad, through interaction with a so-called 'switching' loop close to the active site [45].

Point mutants in the switching loop (at Trp^{285} and Glu^{286}) as well as in the C-terminal peptide (most notably Ile^{1100}) interfere with the activation of the catalytic site and prevent high-affinity ubiquitin binding. Interestingly, USP7 overexpression in cells is capable of stabilizing both MDM2 and p53, but these mutations completely abrogate that ability, showing that the activation is critical for proper functioning in cells [45].

Thus the UBL domains in USP7 function to activate the catalytic domain. They bind to the catalytic domain at a site different from ubiquitin and promote ubiquitin binding, rather than interfering with it. Therefore the activation is UBL-dependent, but not by mimicking ubiquitin function.

Modulation of UBL function

Interestingly, the regulatory roles of UBL domains in both USP4 and USP7 can be modulated further by other proteins. Two examples have been identified (Figure 3), but others may yet be found.

In the spliceosomal Prp19 complex, a second USP, USP39, is found besides USP4 itself [24]. This unusual DUB lacks the catalytic residues and therefore cannot cleave ubiquitin from any target [25], but it does have the conserved ubiquitin-binding site. Interestingly, it also has kept the ability to bind the USP4 internal UBL domain, with similar affinity. When USP39 is added to the USP4 catalytic domain, it can provide a partial relief of USP4 ubiquitin-binding inhibition by the internal UBL domain [29]. So far this has only been analysed *in vitro* and obviously this mechanism needs to be studied in the context of the spliceosome. Moreover, it is possible that other USPs play a similar modulating role, thus requiring further studies.

The USP7 protein is in a dynamic equilibrium, between the activated and the inactive state. This is seen in the SAXS analysis and it could be confirmed by analysis of the effect of the modulating protein GMPS (GMP synthetase) [45].

GMPS is a metabolic enzyme that is important for the synthesis of GMP. However, part of GMPS in cells is found in a complex with USP7 [48–50]. It is capable of activating USP7 against various targets, including p53, and it is essential for the activity of USP7 against histone H2B [48–50]. Thus GMPS clearly has roles in determining target specificity.

However, it could be shown that GMPS also activates USP7 against a minimal target, such as ubiquitin–AMC (7-amino-4-methylcoumarin). It binds to HUBL123 with high affinity and it does not interact with either the catalytic or

the HUBL45 domains. Nevertheless, its ability to activate USP7 requires the proper activation through the HUBL45 domain interaction with the switching loop, since neither USP7 lacking the activating HUBL45 nor USP7 with point mutants in the switching loop or activation peptide can be activated by GMPS. This suggested that GMPS would activate USP7 by shifting the equilibrium between the active and the inactive state. In that case, it should stimulate the interaction between the catalytic domain and the HUBL45 region. Indeed, binding studies showed that addition of GMPS could shift the equilibrium between the catalytic domain and the complete HUBL domain from 50 μM to 2 μM, whereas the HUBL123 does not bind at all under these conditions, indicating that GMPS indeed allosterically stabilizes the activated state.

In this manner, regulatory proteins can change the effect of the UBL-dependent inhibition (USP4) or activation (USP7). It seems likely that other modulators of these functions may exist. In particular, the HUBL domain in USP7 would be a prime target for inactivation, since the equilibrium could provide an excellent manner to (temporarily) attenuate the function of this important DUB.

Future perspectives

Currently, we are starting to gain information about regulatory roles for UBL domains in USP function at the biochemical level. For USP7, the importance of the activation by the C-terminal HUBL domain has been validated in cells, as it was shown that overexpressed USP7 variants with mutations in the switching loop or C-terminal peptide lost the ability to stabilize p53 or MDM2 in cells. In contrast, the recruitment and inhibitory roles of the UBL domains in USP14 and USP4 needs validation.

For most UBL domains in USPs, we do not yet know their function. It will therefore be interesting to see whether these UBL-containing USPs are regulated in similar ways and what, e.g., the effect of the N-terminal UBL domain on USP4 may be. One would imagine that USP11 and USP15 show similar regulatory mechanisms since they are closely related paralogues, despite differences in N-terminal domains and cellular functions.

One interesting question will be whether the localization with respect to the active site is relevant for the regulatory role of the UBL domain. If that is the case, the current studies on USP14, USP4 and USP7 might be predictive for UBL roles in other USPs. Future studies will be required to address this question.

It seems likely that any UBL domain in a USP is subject to further regulation from external modulators, such as has been described in the present article for the proteasome, USP39 and GMPS. One could also envisage possible other modulators which could, e.g., stabilize the inactive state for USP7 or the inhibited state for USP4. Many different interactors of USPs are known [51] and some may well have such functions.

An interesting variation on regulation of USPs by UBL domain is that by UAF1. This regulator of USP1, USP12 and USP46 contains two domains with a ubiquitin or SUMO-like fold. One of these domains is involved in the recruitment to targets [28]. The domain is not a member of the USP family of UBL domains, but it illustrates that further UBL domains can be involved in their regulation and suggests a fourth possible role of these UBL domains.

Finally, it would be of interest to decipher why such similar modules have evolved to these highly different functions and to which extent the ubiquitin scaffold is relevant to their function. Are there any rules at the sequence level or could one predict a function from the location in the USP? These are questions for future research.

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